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## Inducing specific reactivity against B cells in mice by immunizing with an Fc fusion protein containing self-Ig $\beta$

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**Abstract** A recombinant chimeric fusion protein, muIg $\beta$ -h $\gamma$ 4.Fc, composed of the extracellular domain of mouse Ig $\beta$  (CD79b) and the CH2-CH3 domains of human IgG $\gamma$ 4.Fc (h $\gamma$ 4.Fc), linked via an immunologically inert flexible peptide, was prepared. The fusion protein was evaluated for its ability to induce specific auto-reactive immune response against Ig $\beta$  and to modulate B cell activity in Balb/c mice. Upon immunization with muIg $\beta$ -h $\gamma$ 4.Fc, mice developed immunoglobulin (IgG) against self-Ig $\beta$ , which could bind to the cells of a mouse B cell line expressing Ig $\beta$  on the cell surface. The proportion of B cells in mononuclear cells in the peripheral blood (PBMC) of treated mice decreased as compared to that of mice immunized with h $\gamma$ 4.Fc without the Ig $\beta$  component. Furthermore, mice immunized against muIg $\beta$ -h $\gamma$ 4.Fc displayed a reduced antibody response against an irrelevant antigen. The implications of employing the present approach in developing a therapeutic strategy for regulating B cell activity has been discussed.

**Keywords** Auto-reactivity · B cell antigen receptor · Fc fusion protein · Surface antigen Ig $\beta$  (CD79b)

### Introduction

Transmembrane glycoprotein Ig $\beta$  (also known as CD79b or B29) forms a disulfide-linkage with Ig $\alpha$  (CD79a or mb-1) on the cell surface of B lymphocytes. The Ig $\alpha$ /Ig $\beta$  heterodimers are non-covalently associated with membrane-bound immunoglobulins (IgG) of all isotypes in the B cell antigen receptor (BCR) complex [39, 48]. Both Ig $\alpha$  and Ig $\beta$  mediate initial signal

transduction events subsequent to antigen stimulation [42, 33]. The cytoplasmic tails of Ig $\alpha$ /Ig $\beta$  can trigger the signaling of the Fyn, Src, and Syk family kinases, leading to B cell differentiation and proliferation [8, 19, 21]. In addition, mutations or gene knockout of Ig $\alpha$  or Ig $\beta$  can block the development of B cells in humans and mice [3, 14, 27, 36, 45].

Several studies have shown that Ig $\beta$  molecules are expressed early on in the pro-B cell stage, even before IgG gene rearrangement, through to the plasma cell stage [15, 20, 22, 35]. Consequently, Ig $\beta$  has been regarded as a specific surface marker for B-lineage cells. The expression of Ig $\beta$  at mRNA and protein levels has been identified in various B cell lines and in several kinds of human malignancies derived from immature or mature B cells [49]. Thus, the development of monoclonal antibodies against Ig $\beta$  for potential use in targeting B cell tumors has been investigated [22, 52]. Furthermore, using these monoclonal antibodies as B cell suppressants to control B cell activity has also been suggested [32].

The present study was designed to explore the potential of using autologous Ig $\beta$  to induce specific auto-reactive antibodies actively against self-Ig $\beta$ . Since self-antigens are immunologically tolerated, the extracellular domain of mouse Ig $\beta$  was coupled with the CH2-CH3 domains of human IgG $\gamma$ 4.Fc (h $\gamma$ 4.Fc) to form a dimeric fusion protein, muIg $\beta$ -h $\gamma$ 4.Fc. The rationale for this approach was that the autologous Ig $\beta$  within the recombinant protein should provide the antigenic target for B cells that have the receptors specific for Ig $\beta$  but are silent owing to the lack of interacting helper T cells. On the other hand, the foreign human  $\gamma$ 4 CH2-CH3 domains should provide antigenic peptides that, after being processed and presented in association with the MHC class II antigens by the antigen-specific B cells or other antigen presenting cells, can be recognized by specific helper T cells. These helper T cells can then activate the B cells which, having bound, internalized, and processed the fusion protein, would in turn present the antigenic peptides for T cell recognition.

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## Materials and methods

### Construction of the $\text{mulg}\beta\text{-huy4.Fc}$ expression vector

Total RNA from mouse spleen cells was prepared with TRIzol reagent (Gibco BRL, Gaithersburg, Mich.) according to the manufacturer's instructions. First-strand cDNA was synthesized by M-MLV reverse transcriptase (Promega, Madison, Wis.) with oligo(dT) as reverse primer. Nest-polymerase chain reaction (PCR) amplification was performed for the extracellular portion of mouse Ig $\beta$ . Regarding the first primer sets, the forward primer, mIg $\beta$ F1 (5' CATGTTTCGCCCCAAAC 3'), is located in the 5'-untranslated region and the reverse primer, mIg $\beta$ R1 (5' GAGGATGATGAGG-AGGGT 3'), is located within the transmembrane region. In the second PCR, primers were designed to contain restriction endonuclease sites. The forward primer with the *Hind*III site is mIg $\beta$ F2 (5' CCCAAGCTTCATCTACAATGGCCACACTGGTGCTG 3'), and the reverse primer with the *Bam*HI site is mIg $\beta$ R2 (5' CGCGGATCCATCTTTGAGTGTGTTCCG 3'). PCR reactions were performed for 30 cycles as follows: 94°C, 30 s for denaturation; 48°C (for the first PCR) or 60°C (for the second PCR), 30 s for annealing; 72°C, 60 s for elongation. The resultant PCR products were analyzed on a 2% agarose gel. A 370-bp DNA segment, including its native leader sequence, was purified and cloned into human IgG $\gamma$ 4.Fc fusion protein expression vector (a kind gift from S. L. Morrison, UCLA, Calif.). The two moieties of the fusion protein are linked through a T cell immunologically inert peptide, Gly Gly Ser Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser. The DNA sequence of the desired clone was confirmed by the dideoxy chain termination method.

### Transfection and expression of $\text{mulg}\beta\text{-huy4.Fc}$ expression vector

Adherent cultures of Chinese hamster ovary/dhfr<sup>-</sup> cells (CHO/dhfr<sup>-</sup>; ATCC) were maintained at 37°C in IMDM culture medium (Gibco BRL, Gaithersburg, Mich.) with 10% fetal bovine serum (FBS). Purified  $\text{mulg}\beta\text{-huy4.Fc}$  expression vector was transfected into CHO/dhfr<sup>-</sup> cells using transfectamine (Clontech, Palo Alto, Calif.) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were divided into 96-well dishes and changed to selection medium containing 600  $\mu\text{g}/\text{ml}$  G418 (Calbiochem, Rtecs, Mass.). The contents of expressed protein in the supernatants were measured by anti-human IgG specific sandwich enzyme-linked immunosorbent assay (ELISA). The first coated antibodies were goat anti-human IgG antibodies (KPL, Gaithersburg, Md.), and the secondary antibodies were mouse anti-human IgG antibodies (ICN, Aurora, Ohio). The high-yield single-cell clones (> 10  $\mu\text{g}/\text{ml}$ ) were selected and expanded for protein expression. Further transfected gene amplification in the stable transfectant was achieved by continuous treatment with methotrexate (MTX; Calbiochem, Rtecs, Mass.). To prevent possible contamination of bovine IgG in purified proteins, the sera for culture medium were subjected to protein A plus G-Sepharose affinity chromatography (Amersham Pharmacia, Piscataway, N.J.) before use.

### Purification and characterization of the $\text{mulg}\beta\text{-huy4.Fc}$ fusion protein

The  $\text{mulg}\beta\text{-huy4.Fc}$  fusion protein from the supernatants was purified by protein A-Sepharose affinity chromatography (Amersham Pharmacia, Piscataway, N.J.). After absorption and washing, the fusion protein was eluted with 100 mM glycine (pH 2.5) and neutralized in 1.5 M Tris-HCl buffer (pH 8.5). Fractions containing the  $\text{mulg}\beta\text{-huy4.Fc}$  fusion protein were concentrated and stored in phosphate-buffered saline (PBS) containing 50% glycerol at -20°C before use. The purified proteins were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For further

characterization, the purified proteins were analyzed by western blotting analysis with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibodies (KPL, Gaithersburg, Md.).

### Immunization of Balb/c mice

Five to 6-week-old Balb/c mice were purchased from the National Laboratory Animal Breeding and Research Center (NLABRC, Taiwan) and maintained under pathogen-free conditions. Eight to 9-week-old mice were primed i.p. with  $\text{mulg}\beta\text{-huy4.Fc}$  fusion protein in complete Freund's adjuvant (Sigma ImmunoChemicals, St. Louis, Mo.) at a dosage of 50  $\mu\text{g}/\text{mouse}$ . The treated mice were boost-immunized with recombinant protein in incomplete Freund's adjuvant every 2 weeks. Control groups were treated with either PBS as a negative control, or equal amounts of human IgG $\gamma$ 4.Fc as a positive control in Freund's adjuvant. For B cell activity assay, the ovalbumin protein (OVA; Sigma, St. Louis, Mo.) was given with complete Freund's adjuvant after 1 week of the fourth immunization of  $\text{mulg}\beta\text{-huy4.Fc}$  fusion protein at a dosage of 20  $\mu\text{g}/\text{mouse}$ . The second and third OVA immunizations were boosted with incomplete Freund's adjuvant 1 week after the fifth and sixth immunizations of  $\text{mulg}\beta\text{-hulG.Fc}$  fusion protein, respectively.

### Determination of antigenic specificity from immunized mice by ELISA

The antigenic specificity of anti-self Ig $\beta$  auto-reactive antibodies was detected using an ELISA. Solid-phase antigens were human IgG, Mi $\beta$ P-mul $\beta$ , or MBP. After blocking with 5% non-fat milk, antisera collected from immunized mice 1 week after each boost-immunization were added at various dilutions to the plates and incubated for 2 h at 37°C. Sera prior to immunization (pre-bleed) were used as a negative control. Specific binding was detected using HRP-conjugated goat anti-mouse IgG antibodies (ICN, Aurora, Ohio) and TMB substrate (Sigma, St. Louis, Mo.) for color development. The reaction was stopped by adding 1 M H<sub>3</sub>PO<sub>4</sub>, and absorption at optical density (OD) 450 nm was determined by a Titertek Multiskan ELISA reader. For the B cell function assay, the ELISA assay using OVA as solid-phase antigen was carried out in the same manner as the procedure described above.

### Western blotting analysis of antisera

A culture of mouse IgM-expressing B cell line, BALENLM-17, was kindly donated by J. T. Kung (IMB, Academia Sinica, Taiwan), and maintained in DMEM culture medium containing 10% FBS and 2.5 mM hypoxanthine/thymidine (Gibco BRL, Gaithersburg, Mich.). The cell line has been identified to express Ig $\beta$  as a B cell surface marker [18].  $1 \times 10^7$  cells were washed with ice-cold PBS twice and lysed in 1 ml cell-homogenization buffer (10 mM Tris-HCl at pH 7.4, 50 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 1 mM benzamide, 0.5  $\mu\text{g}/\text{ml}$  aprotinin, and 50 mM NaF). The cell lysate proteins (50  $\mu\text{g}/\text{well}$ ) were separated in 10% SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, Mass.) with CAPS buffer (Sigma, St. Louis, Mo.). The membranes were cut into several strips, and each strip was placed in one well. After blocking with 5% non-fat milk, the strips were hybridized with antisera at a dilution of 1:500 followed by incubation with HRP-conjugated goat anti-mouse IgG antibodies. The specific protein bands were visualized using TMB substrates.

### Characterization of antisera by cell surface binding assays

$1 \times 10^6$  BALENLM-17 cells were suspended in 100  $\mu\text{l}$  5% fetal bovine serum (FBS) in Hanks' buffered-saline solution (HBSS; Gibco BRL, Gaithersburg, Mich.) and 1  $\mu\text{l}$  antisera from immu-

nized mice. After staining on ice for 30 min, the cells were washed twice with HBSS and suspended in 100  $\mu$ l 5% FBS in HBSS containing FITC-conjugated goat anti-mouse IgG antibodies (BD, San Diego, Calif.). For the analysis of fluorescent staining, cells were analyzed with a FACScan instrument (Becton Dickinson, San Jose, Calif.) and CellQuest software. Cells were gated for forward and side scatter analyses.

#### In vivo effects of muIg $\beta$ -huy4.Fc fusion protein on B/T lymphocytes

About 100  $\mu$ l of blood was collected from each immunized mouse 1 week after each boost-immunization, and mixed with 10 ml ice-cold RBC lysis buffer (150 mM NH<sub>4</sub>Cl, 15 mM KHCO<sub>3</sub>). Lymphocytes were washed twice with RBC lysis buffer and double-stained with FITC-conjugated rat anti-mouse  $\kappa$  (for B cells in FL1; kindly donated by J. T. Kung, IMB, Academia Sinica, Taiwan) plus PE-conjugated goat anti-mouse CD3 (for T cells in FL2) antibodies (BD, San Diego, Calif.). Fluorescent staining on the lymphocytes was analyzed with a FACScan by gating lymphocytes on the basis of forward and side scatter analyses as well as lymphocyte marker expression. The blood from the mice injected with PBS alone was used as a control.

## Results

### Antigenic specificity of antisera from muIg $\beta$ -huy4.Fc immunized mice

To characterize the antigenic specificity of antisera from mice immunized with muIg $\beta$ -huy4.Fc, an ELISA with a fusion protein, maltose binding protein-mouse Ig $\beta$  (MBP-muIg $\beta$ ) as solid-phase antigen, was performed. Specific activity could be detected after the second

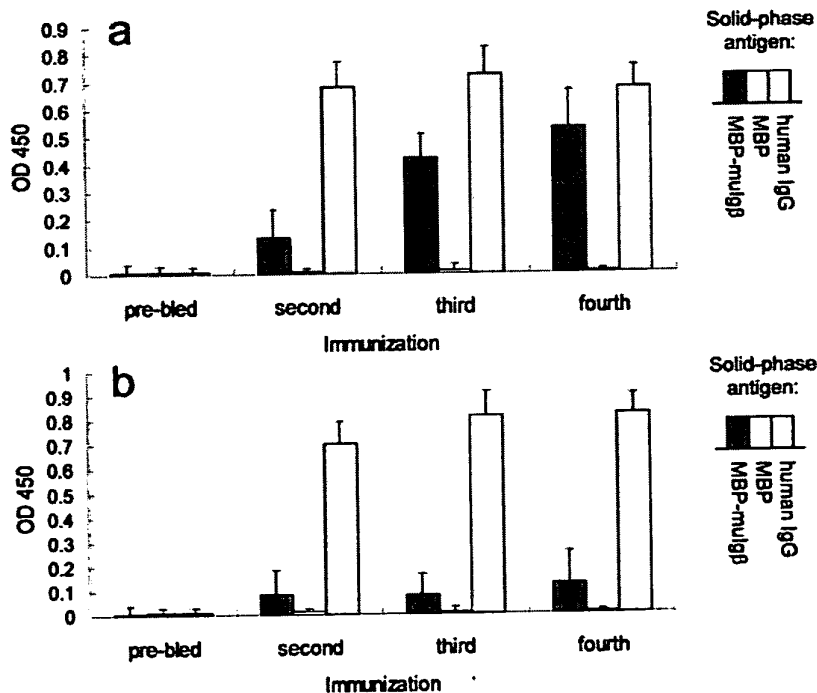
immunization of the recombinant muIg $\beta$ -huy4.Fc protein, which became more pronounced with the third and fourth immunizations (see Fig. 1a). Antisera from mice immunized with huy4.Fc showed a marginal increase in OD, although this was not statistically significant, against MBP-muIg $\beta$  (Fig. 1b). None of the sera from the control mice injected with PBS alone contained the auto-reactive antibody against mouse Ig $\beta$  (data not shown). None of the antisera tested in this study reacted to MBP alone.

These antisera were also tested by western blotting analysis with IgM-expressing BALENLM-17 cell (a Balb/c B cell lymphoma) lysates. Under reducing conditions, antisera from immunized mice showed reactivity against three protein bands of 47, 38, and 34 kDa, respectively (Fig. 2; lanes 3–5). These three bands may correspond to the three kinds of glycosylation patterns on Ig $\beta$  that have been identified in previous works [12, 32, 47]. In addition, there is an unknown 68 kDa band that requires further characterization. No significant protein band was detected when the protein strips were hybridized with the fourth antisera from mice immunized with huy4.Fc (Fig. 2; lane 6) or treated with PBS alone (Fig. 2; lane 7).

### Cell surface binding of antisera from immunized mice

To determine whether the induced auto-reactive antibodies could recognize the native structure of Ig $\beta$ , BALENLM-17 cells were stained with antisera. After

**Fig. 1a, b** Specific reactivity of the antisera against mouse Ig $\beta$  in ELISA. The solid-phase antigens were MBP-muIg $\beta$ , MBP, or human IgG (for huy4.Fc). Data were from two separate experiments. In each experiment, 15 mice were immunized with a muIg $\beta$ -huy4.Fc fusion protein or b huy4.Fc (as control). The values (means  $\pm$  SD) of OD 450 nm absorption represent the results at a dilution of 1:1,000



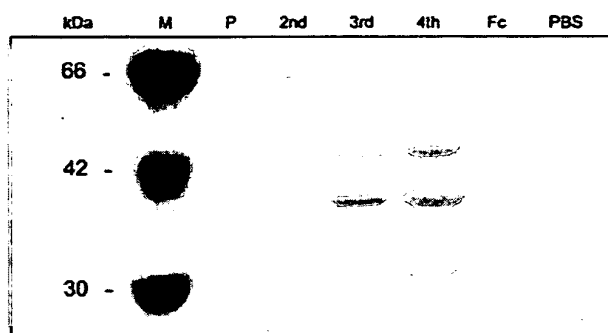


Fig. 2 Western blotting analysis of the antisera from treated mice. The membrane strips, which contained mouse Ig $\beta$  protein and were prepared as described in Materials and methods, were hybridized with antisera at 1:500 dilution. Lane 1 (labeled M): protein markers; lane 2 (P): sera prior to immunization; lanes 3, 4 and 5: antisera after the second, third and fourth immunizations of muIg $\beta$ -huy4.Fc, respectively; lane 6 (Fc), antisera from the fourth immunization of huy4.Fc; lane 7 (PBS), sera from mice treated with PBS

the second immunization with the recombinant protein the antisera from the treated mice could bind to the B cell surface, as shown by fluorescence flow cytometry (Fig. 3a). The subsequent third and fourth immunizations were able to sustain the antisera binding to BALENLM-17 cell surface (data not shown). In contrast, the sera from control mice immunized with huy4.Fc could not stain the BALENLM-17 cell surface, showing that auto-reactive antibodies could not be induced by huy4.Fc immunization (Fig. 3b). Sera prior to the immunization procedure (pre-bled) were used as a negative control.

The non-specific binding of huy4.Fc antisera in FACS should increase after several immunizations with Freund's adjuvant. One probable explanation for the absence of this observation is that in the FACS analysis the cell suspensions, antisera, and the secondary antibody were all blocked with 5% FBS in HBSS. In addition, the secondary antibody, FITC-conjugated goat anti-mouse IgG antibody, was able to cross-react slightly with IgM-expressing Bal-17 cells, raising the comparative background levels of the negative controls and hence masking the reactivity of the huy4.Fc antisera.

#### Reduction of B cell levels in mice producing auto-reactive anti-Ig $\beta$ antibodies

To determine whether the induced auto-reactive antibodies against autologous Ig $\beta$  could influence the total B lymphocyte levels, peripheral blood was collected from each immunized mouse 1 week after each boost-immunization. The lymphocytes in the blood were double-stained with FITC-conjugated anti- $\kappa$  (for B cells in FL1) and PE-conjugated anti-CD3 (for T cells in FL2) monoclonal antibodies (Fig. 4). The results of fluorescence flow cytometry showed that after eliciting auto-reactive antibodies against Ig $\beta$  in treated mice, the ratios of B to T lymphocytes declined (Fig. 4a) compared to the

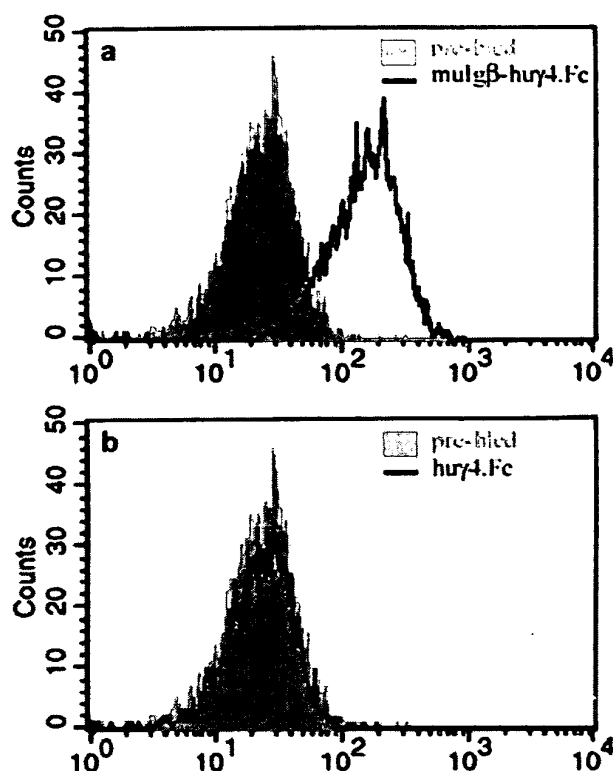


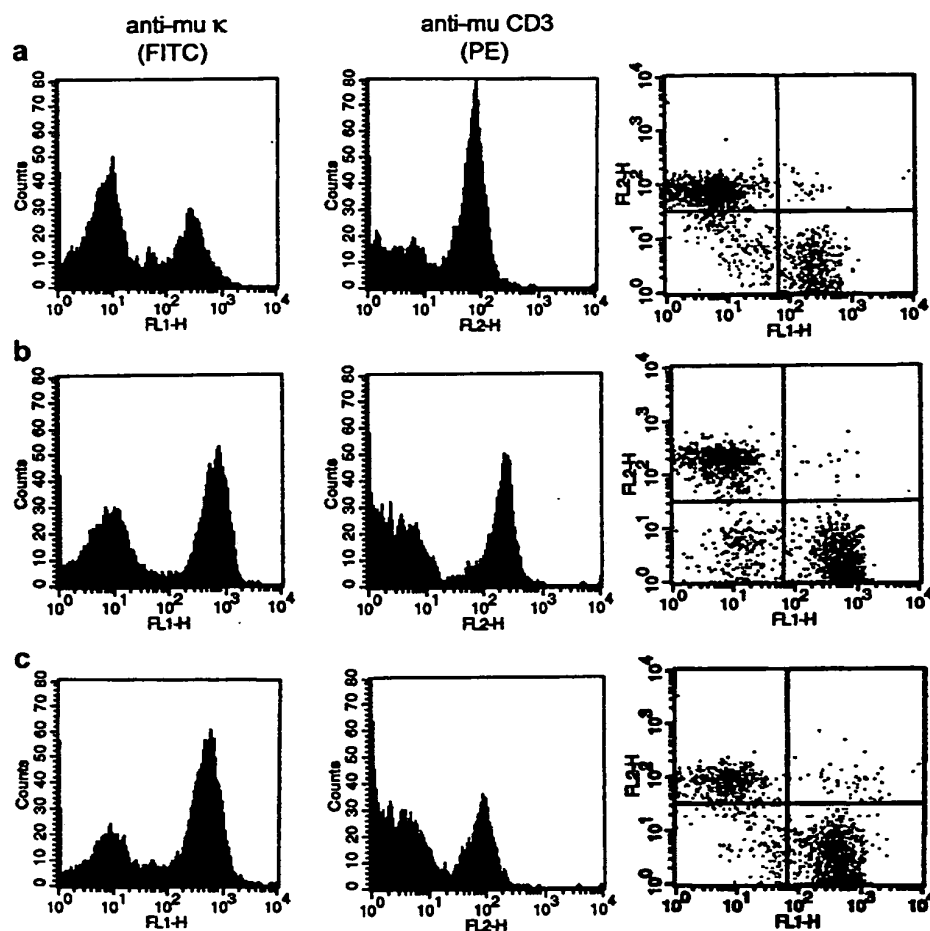
Fig. 3a, b Binding to B lymphoma cells of antisera from muIg $\beta$ -huy4.Fc immunized mice.  $1 \times 10^6$  BALENLM-17 cells were stained with antisera from mice immunized with a muIg $\beta$ -huy4.Fc or b huy4.Fc. Pooled sera from mice prior to muIg $\beta$ -huy4.Fc immunization (pre-bled) were used as a negative control in both a and b. The antisera were obtained after the second immunization with the respective antigens

corresponding ratios in control mice injected with PBS alone (Fig. 4b). The levels of B cells *in vivo* decreased significantly to 31% of total mononuclear cells after the fourth immunization, but increased to about 59% when the mice were analogously immunized with huy4.Fc (Fig. 4c and Table 1). The increased levels of B cells in the latter mice showed the typical B cell expansion after mounting a rigorous immune response.

#### Down-regulation of B cell activity in mice immunized with muIg $\beta$ -huy4.Fc

To investigate whether muIg $\beta$ -huy4.Fc immunizations could also control the activity of the targeted B cells, the treated mice were immunized with an irrelevant antigen, ovalbumin (OVA), in Freund's adjuvant. After the third immunization with OVA, the level of anti-OVA activity was assessed by ELISA (Fig. 5). The antibody response against OVA in mice pre-treated with huy4.Fc was as strong as that in mice pre-treated with PBS. A significant reduction in the humoral immune response against OVA, approximately 50% of that observed in the control, was

**Fig. 4a-c** Reduction of B cell levels in mice producing auto-reactive anti-Ig $\beta$  antibodies. PBMC were collected from treated mice 1 week after the fourth immunization. B and T cells were distinguished by double-staining with FITC-conjugated rat anti-mouse  $\kappa$  (for B cells in FL1) and PE-conjugated goat anti-mouse CD3 (for T cells in FL2) antibodies. PBMC were collected from a mice immunized with mulg $\beta$ -huy4.Fc fusion protein; b mice injected with PBS; or c mice immunized with huy4.Fc



**Table 1** The proportions of cells in the four sectors in the dot-plots of Fig. 4 as percentages of mononuclear cells in the peripheral blood of treated Balb/c mice<sup>a</sup>

Sector	mulg $\beta$ -huy4.Fc			huy4.Fc	PBS
	2nd	3rd	4th		
$\kappa^+/\text{CD}3^+$	1.2 $\pm$ 0.3	1.3 $\pm$ 0.4	1.3 $\pm$ 0.2	1.6 $\pm$ 0.4	1.0 $\pm$ 0.3
$\kappa^+/\text{CD}3^-$	33.2 $\pm$ 6.2	38.9 $\pm$ 7.3	31.2 $\pm$ 6.7	59.4 $\pm$ 9.6	46.4 $\pm$ 5.4
$\kappa^-/\text{CD}3^+$	57.2 $\pm$ 6.8	50.6 $\pm$ 9.0	58.2 $\pm$ 7.1	30.5 $\pm$ 8.2	42.5 $\pm$ 6.5
$\kappa^-/\text{CD}3^-$ , (mean $\pm$ s.d.)	8.4 $\pm$ 2.9	9.2 $\pm$ 2.3	9.3 $\pm$ 1.8	8.5 $\pm$ 2.5	10.1 $\pm$ 2.1
P value (cf. PBS)	0.0007	0.0334	< 0.0001	0.0047	—
P value (cf. huy4.Fc)	0.0002	0.0024	< 0.0001	—	—

<sup>a</sup>The controls are from mice immunized with huy4.Fc or treated with PBS four times. Data are means and standard deviations (SD) of results from 28–30 mice per treatment

found in mice that had been pre-treated with the recombinant mulg $\beta$ -huy4.Fc fusion protein. These results show that the response to irrelevant antigens is reduced in mice pre-treated with mulg $\beta$ -huy4.Fc protein, probably due to a reduction in the levels of B cells.

#### Increased proportion of B cells after termination of mulg $\beta$ -huy4.Fc immunization

After the fourth immunization, the treated mice did not receive the mulg $\beta$ -huy4.Fc fusion protein for 2 months;

and the specific activity of anti-Ig $\beta$  in blood declined after the last immunization with the recombinant protein (Fig. 6). The antisera bound weakly to BALE-NLM-17 cells compared with antisera that were collected 1 week after the last immunization (data not shown). The proportion of B cells in total mononuclear cells returned to about 43%, close to the normal level of about 47% (data not shown). These results show that the specific auto-reactivity against Ig $\beta$  was induced and maintained only via continual immunizations with recombinant mulg $\beta$ -huy4.Fc fusion protein.

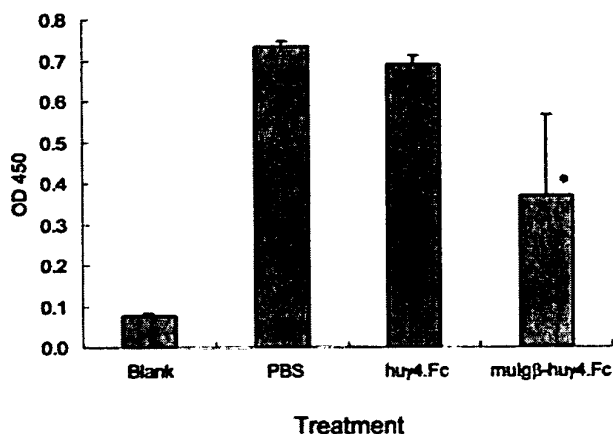


Fig. 5 Down-regulation of B cell activity in mice immunized with mulgβ-huy4.Fc. After the fourth immunization with mulgβ-huy4.Fc, the mice were injected with OVA in Freund's adjuvant as described in Materials and methods. The antisera after the third immunization of OVA were tested by ELISA at a dilution of 1:1,000. *Blank*: sera from mice injected with only PBS; *PBS*: antisera from mice injected with PBS followed by OVA; *huy4.Fc*: antisera from mice immunized with huy4.Fc followed by OVA; *mulgβ-huy4.Fc*: antisera from mice immunized with mulgβ-huy4.Fc followed by OVA. Data are means  $\pm$  SD of results from 30 mice. \*The *p* value from the comparison between the data for mulgβ-huy4.Fc and huy4.Fc was 0.0076

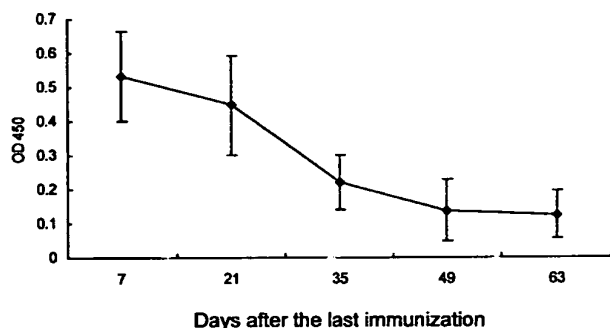


Fig. 6 Reactivity with Igβ of sera after termination of mulgβ-huy4.Fc immunization. After the fourth immunization with mulgβ-huy4.Fc, sera were obtained at the indicated time points, and reactivity with MBP-mulgβ was assessed by ELISA at a dilution of 1:1,000. Each number represents the mean  $\pm$  SD of results from six mice

## Discussion

In this study, Balb/c mice were used as an animal model to induce a specific auto-reactive immune response against their Igβ by immunization with a mulgβ-huy4.Fc recombinant protein. Auto-reactive B cells have been found not only in patients with autoimmune disease, but also in healthy individuals [7, 11, 38]. These auto-reactive B cells can take up autologous antigens through their BCR complex and express the processed peptides on MHC II molecules [24, 40]. However, no appropriate

T helper (CD4<sup>+</sup>) cells could recognize the MHC-presented self-peptides, resulting in the failure to activate auto-reactive B cell clones [1, 4, 5]. This immune regulation can maintain B cell tolerance toward self-antigens. To circumvent this tolerance, special means must be devised to induce auto-reactivity that is both sufficiently strong and specific against a self-antigen.

In our study, specific auto-reactive IgG against autologous Igβ was found to be actively induced in mice immunized with mulgβ-huy4.Fc through a T cell dependent immune response. These auto-reactive antibodies appear to recognize epitopes on Igβ molecules, thus influencing B lymphocyte levels in mice. Compared with a typical immune response, which results in increased B lymphocyte levels, the recombinant protein was found to exhibit negative-regulatory activity in B-cell-related humoral immunity. Significant suppression of antibody activity against an irrelevant antigen was found, indicating a reduction of B cell activation/expansion.

The reason that we used a BCR antigen as the B cell marker was based on the fact that membrane IgGs could be found at a very early stage of B cell development through the terminal differentiated plasma cells. BCR should constitute a rational marker for monitoring B cell responses. However, one possible complication was that the induced anti-self Igβ auto-antibodies in the treated mice might have down-regulated BCR expression on B cells and did not actually affect B cell levels. Based on the data presented in Fig. 4 and Table 1, the percentage of cells in the  $\kappa^-/CD3^-$  sector did not significantly increase after the elicitation of auto-antibodies, indicating that the decreased levels of  $\kappa^+/CD3^-$  B cells were not due to the possible down-regulation of BCR expression. Another relevant point was that the  $\lambda$  light chain-bearing B cells account for only 10% of the B cell population in Balb/c mice. Based on these considerations, anti- $\kappa$  mAb was chosen among the several antibodies that could be used to stain B cells.

Not only can the triggered auto-reactive antibodies down-regulate total B cell activity, but they can also probably feedback-control its own production. After the fifth immunization with the recombinant protein, the specific activity against self-Igβ was found to have decreased (data not shown). It is known that autoantigen selection/activation resulting in the production of auto-reactive antibodies plays an important role during the pathogenesis of antibody-mediated autoimmune diseases. The potential use of recombinant proteins such as mulgβ-huy4.Fc in down-regulating B cell activity therefore warrants further investigation.

It is not clear how the induced auto-reactive antibodies participate in immune regulation. Certain monoclonal antibodies that are effective in treating B cell lymphoma, such as anti-idiotypic antibodies that bind to BCR of certain lymphoma cells or Rituximab (anti-CD20), have been found not only to recruit effectors but also to achieve their cytotoxic effects using direct signaling mechanisms [43, 46, 50]. They appear to



cross-link the target molecules and deliver transmembrane signals that control cell division [2, 13]. Cell targeting by antibody-dependent cell-mediated cytotoxicity (ADCC) or apoptosis by crosslinking of signal-delivering Ig $\beta$  could be possible events following the elicitation of anti-Ig $\beta$  auto-reactive antibodies.

Other methods have also been developed to break down immunological tolerance [16, 17, 28, 29, 37, 41]. Homologous protein mimicry and insertion of a T cell epitope into a self-antigen sequence have been shown to induce auto-reactive/cross-reactive immunity with designed specificity that can eliminate certain cell types [9, 23, 25, 26, 30, 51]. In homologous protein mimicry, activation of auto-reactive B cells by antigenic mimicry from homologous foreign antigens could allow the priming of auto-reactive T cells. Through this mechanism of breaking down T cell self-tolerance, endogenous antigens could lead to sustained antibody production in the absence of the foreign antigen [25, 26]. On the other hand, insertion of a T cell epitope into a self-antigen may influence the tertiary structures and immunogenicity of the autologous antigen [4, 5, 6]. Novel epitopes consisting of inserted foreign sequence flanking self-sequence may also allow the priming of auto-reactive T cells [4, 6].

In our study design, the two moieties of the  $\mu$ Ig $\beta$ -huy4.Fc recombinant protein are linked through a T cell immunologically inert peptide, Gly-Gly-Ser-Gly-Gly-Ser-Gly Gly-Gly-Gly-Ser-Gly-Gly-Gly-Ser [44]. The insertion of this peptide at the fusion point can minimize possible neoantigenicity created by the joining of the two moieties. Furthermore, the Fc component is an attractive fusion partner because of its ease of quantification by ELISA and purification by protein A affinity chromatography. It can help to dimerize the recombinant chains, which in some cases may stabilize the constructs and enhance binding and biological activities. In addition, Fc of certain classes can extend the half-lives of the protein moieties of interest and enhance the uptake by antigen-presenting cells when binding to Fc receptors on these cells. Owing to these advantages, Fc fusion proteins have been explored and developed for therapeutic purposes [10, 31, 34, 53].

This study appears to be the first attempt to explore the potential of inducing specific auto-antibodies against B cells. Our results present an interesting twist, since the production of auto-antibodies requires the presence of B cells. In subsequent studies, we are investigating how the immunogenicity of  $\mu$ Ig $\beta$ -huy4.Fc can be enhanced in Balb/c mice. We are also studying the ability of Fc fusion proteins containing other B cell specific antigens, such as Ig $\alpha$  (CD79a) and CD20, to induce a specific immune response against B cells.

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